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Propionic and methylmalonic acids increase cAMP levels in slices of cerebral cortex of young rats via adrenergic and glutamatergic mechanisms

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Abstract

We have previously described that propionic (PA) and methylmalonic (MMA) acids increased the in vitro phosphorylation of cytoskeletal proteins through cAMP-dependent protein kinase and glutamate. In the present study we investigated the in vitro effects of 1 mM glutamate, 2.5 mM MMA and 2.5 mM PA on cAMP levels in the slices of cerebral cortex of young rats. Results showed that PA, MMA and glutamate increased cAMP levels after 30 min of incubation, while the β-adrenergic agonist epinephrine elicited a similar effect only at a shorter incubation time. Then effects were prevented by the β-adrenergic antagonist propranolol, rather than by glutamate antagonists (AP5, CNQX and MCPG), suggesting that they were mediated by β-adrenergic receptors. In addition, glutamate antagonists per se induced increased cAMP levels; however propranolol prevented only the effect elicited by the metabotropic glutamate antagonist MCPG. Taken together, it is feasible that PA and MMA increase cAMP synthesis via a β-adrenergic/G protein coupled pathway, in a glutamate-dependent manner. Although additional studies will be necessary to evaluate the importance of these observations for the neuropathology of propionic and methylmalonic acidemias, it is possible that high brain cAMP levels may contribute to a certain extent to the neurological dysfunction of the affected individuals.
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1. Introduction

Methylmalonic acidemia (MMAcidemia) and propionic acidemia (PAcidemia) are inherited neurometabolic disorders of the propionate pathway biochemically characterized by tissue accumulation of methylmalonic acid (MMA) and propionic acid (PA), respectively. Although neurologic symptoms and cerebral atrophy are the most prominent findings of these disorders, very little is known on the pathophysiology of the neurological dysfunction in these disorders. Recently we have demonstrated that MMA and PA, at the concentrations found in tissues of propionic and methylmalonic acidemic children, increase the phosphorylation of Triton insoluble cytoskeletal proteins in the cerebral cortex of rats via *N*-methyl-D-aspartic acid (NMDA) glutamate receptors [1], and that this effect is mediated by cAMP- and Ca^{2+}/calmodulin-dependent protein kinases (PKA and PKCaM) [2]. However, to date, we have not examined the intricate mechanisms involved in such effects.

Adenylyl cyclases (AC) regulate many cell processes in response to intra and extracellular signals such as hormones, neurotransmitters and Ca^{2+} in the central nervous system [3]. The isoforms of adenylyl cyclases are stimulated by the heterotrimeric G proteins, so ligands acting via G protein-coupled receptors (GPCRs) are able to directly activate the adenylyl cyclase/cAMP/PKA pathway.

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This pathway may lead to cellular responses through the phosphorylation of many target proteins [4]. Immunohistochemical studies of adenylyl cyclase in the brain have shown a predominant concentration at synapses [5]. Consistent with this localization, the activation of adenylyl cyclase has been shown to enhance synaptic transmission in hippocampal [6–8] and striatal [9] regions. Thus, cAMP-dependent effects on synaptic transmission have been implicated in long-term potentiation in the hippocampus [8,10,11]. Furthermore, the targeting of PKA to the cytoskeleton is involved in the regulation of signal transduction events. These signals influence fundamental cell properties, such as shape, movement and division [12]. Furthermore, there is evidence indicating that an important extracellular signal activating the cAMP-dependent pathway occurs via β-adrenergic receptors, which are stimulated by noradrenaline [13].

The β-adrenergic receptors belong to the family of G-protein coupled receptors (GPCRs). They are subdivided into the α1, α2, β1, β2 and β3 subtypes, which can be distinguished by adrenergic agonists and antagonists [14,15] and by DNA analysis [16]. A fourth β-adrenoceptor subtype appears to modulate the human heart function [17].

It has been fairly well established that the modulation of cell signaling through membrane receptors, G proteins and other components frequently takes place through phosphorylation/dephosphorylation cycles [18–20]. Many GPCRs are desensitized via feedback regulation by second messenger-stimulated kinases and there is evidence that protein kinase A (PKA) phosphorylates and desensitizes β2-adrenergic receptors [21–23].

Glutamate receptors are subdivided into two groups, ionotropic (iGLURs) and metabotropic (mGLURs) receptors [24]. iGLURs contain cation-specific ion channels and are subdivided into NMDA (*N*-methyl-D-aspartate) and non-NMDA receptors [(*R*,*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid] (AMPA) and kainate receptors. mGLURs are classified into three groups consisting of at least eight subtypes. Group II mGLURs ($mGLU_2$ and $mGLU_3$ subtypes) are thought to be negatively coupled to AC via G_i inhibiting the production of cAMP; group I mGLURs ($mGLU_1$ and $mGLU_5$ subtypes) are coupled to G_q and activate the diacylglycerol–phospholipase C (DAG–PLC) pathway eliciting phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization. Group III mGLURs are also negatively coupled to cAMP and consist of $mGLU_4$ and $mGLU_{6–8}$ subtypes [25].

Taking into account the increasing evidence in the literature of a cross-talk between GPCR-mediated glutamatergic and adrenergic systems [26], in the present study we investigated the effects of PA and MMA on brain cAMP levels. We provided evidence that PA and MMA at concentrations found in PAcidemia and MMAcidemia are able to increase intracellular cAMP levels via adrenergic receptors in a glutamate-dependent manner.

2. Materials and methods

2.1. Radiochemicals and compounds

Cyclic AMP, propionic acid, methylmalonic acid, L-glutamate, NMDA antagonist D-2-amino-5-phosphonopentanoic acid (DL-AP5), beta-adrenoceptor agonist (±)-epinephrine hydrochloride, partial beta-adrenoceptor antagonist (*S*)-(−)-propranolol hydrochloride, benzamidine, leupeptin, antipain, pepstatin, chymostatin, bovine serum albumin, and protein kinase 3′,5′ cyclic AMP dependent were obtained from Sigma (St. Louis, MO).The potent competitive non-NMDA iGluR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and the non-selective group I/group II mGluR antagonist (*R*,*S*)-α-methyl-4-carboxyphenylglycine (MCPG) were purchased from Tocris Neuramin (Bristol, UK). [^{3}H]cyclic AMP (23 Ci/mmol) was from Amersham International (UK). All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

2.2. Animals

Wistar rats (17 days of age) were obtained from our breeding stock. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature (22 °C) colony room. Free water and a 20% (w/w) protein commercial chow were provided. The “Principles of laboratory animal care” (NIH publication No. 85-23, revised 1985) was followed for all the experiments.

2.3. Preparation of tissue slices

Rats were killed by decapitation, and the cerebral cortex was rapidly removed and placed in ice-cold Krebs–Hepes (KR) containing: 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na–HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$, and the protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin, 0.7 μM chymostatin. Slices (400 μm) were rapidly prepared, using a Mcllwain Tissue Chopper.

2.4. Incubation of cerebral cortex slices

Two cortical slices (400 μm) were preincubated in 500 μL of KR at 37 °C for 60 min, the KR being changed twice during this period. In some experiments tissue slices were incubated in KR without $CaCl_2$ in the absence of Ca^{2+} chelators and ionic replacers. When specified, 100 μM DL-AP5, 50 μM CNQX or 100 μM MCPG (glutamate antagonists) was added to the medium 10 min before incubation. Incubation was then started by adding the drugs during different times (5, 15 and 30 min). When indicated, 1 mM glutamate, 10 μM epinephrine, 10 μM propranolol, 2.5 mM PA or MMA (pH 7.2–7.4) were added to the incubation medium.

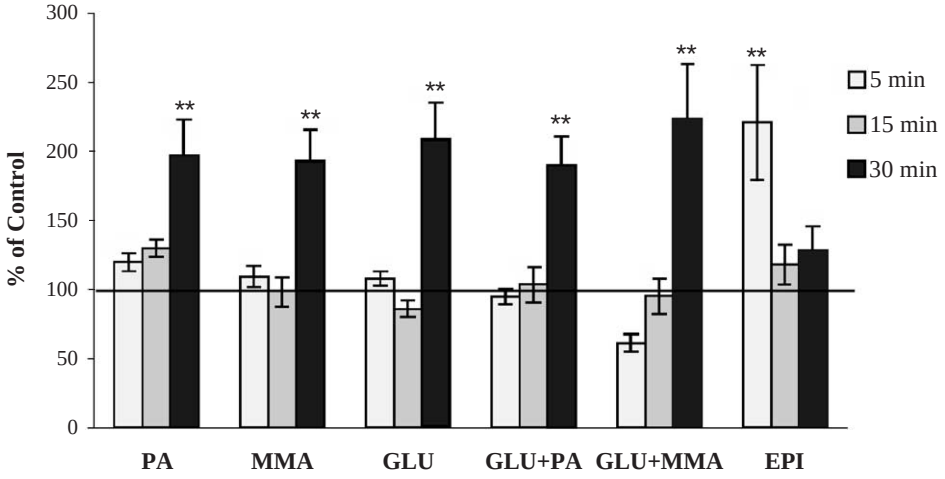

Fig. 1. Effect of in vitro treatment with propionic (PA) or methylmalonic (MMA) acids, glutamate (GLU) and epinephrine (EPI) on cAMP levels in slices from the cerebral cortex of 17-day-old rats at different incubation times. Slices from cerebral cortex were incubated for 5, 15 or 30 min with 10 μM EPI, 2.5 mM PA or 2.5 mM MMA in the presence or absence of 1.0 mM GLU, as described in Materials and methods. Results were calculated as pmol cAMP/mg protein and expressed as percentage of control for 8–10 animals in each group. Data are reported as means±S.E. Statistically significant differences from controls, as determined by one-way ANOVA followed by LSD test, are indicated: $^{**}P<0.001$.

Incubation was stopped by placing the tubes in an ice-cold bath and samples were processed as previously described [27].

2.5. Measurement of cyclic AMP levels

In brief, the incubation medium was replaced by 0.5 N perchloric acid. Slices were homogenized and an aliquot of the homogenate was used for protein measurement by the method of Lowry et al. [28], using bovine serum albumin as standard. The rest of the homogenate was centrifuged at 12,800×*g* for 2 min, the pellet was discarded and the supernatant was neutralized with 2 M KOH and 1 M Tris/HCl. This neutralized supernatant was centrifuged for 3 min at 12800×*g* and an aliquot from the supernatant was evaporated under a stream of air in a 50 °C bath according to a modification of the procedure of [29]. Residues were dissolved in 50 mM Tris–HCl, pH 7.4, containing 4 mM EDTA. Cyclic AMP content was measured by the protein binding method of Tovey et al. [30], using [^{3}H] cyclic-AMP (23 Ci/mmol) and protein kinase 3′,5′ cyclic AMP dependent as the binding protein. In the experiments using glutamatergic and adrenergic antagonists we used purified protein kinase A from bovine heart. Radioactivity was counted by liquid scintillation.

2.6. Statistical analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by LSD when the *F*-test was significant. All analyses were performed using the SPSS (Statistical Package for the Social Sciences) software program in an IBM-PC compatible computer. Each experiment was carried out in triplicate.

3. Results

We first observed that basal cAMP levels in slices from cerebral cortex of 17-day-old rats were not significantly altered at the different incubation times (5–30 min) used in this study, although there was a slight and progressive decrease of these levels as incubation advanced (mean±S.E., *n*=16): 7.36±0.60 pmol/mg of protein at 5 min; 6.73±0.70 pmol/mg of protein at 15 min; and 5.88±0.91 pmol/mg of protein at 30 min.

In order to establish a time-dependent effect of the metabolites accumulating in PAcidemia and MMAcidemia on the cAMP levels, slices of cerebral cortex were incubated with PA or MMA during 5, 15 and 30 min (Fig. 1). We can observe in the figure that PA and MMA increased cAMP levels only after 30 min exposure and that this effect was

Table 1
Effect of propionic and methylmalonic acids, glutamate and glutamate antagonists on cAMP levels in tissue slices incubated in a Ca^{2+}-free buffer

	PA	MMA	GLU	AP5	CNQX	MCPG
cAMP levels (% of control)	101.6±14.6 (7)	109.8±13.5 (7)	105.2±10.8 (9)	94.8±10.6 (8)	97.1±8.3 (4)	90.8±7.3 (4)

cAMP levels were calculated as pmol/mg protein. Results were reported as mean±S.E. No significant differences between the groups was detected at 30 min incubation (one-way ANOVA).

Propionic acid=PA; methylmalonic acid=MMA; glutamate=GLU; NMDA ionotropic glutamate antagonist=AP5; non-NMDA ionotropic glutamate antagonist=CNQX; metabotropic glutamate antagonist=MCPG; () animals in each group.

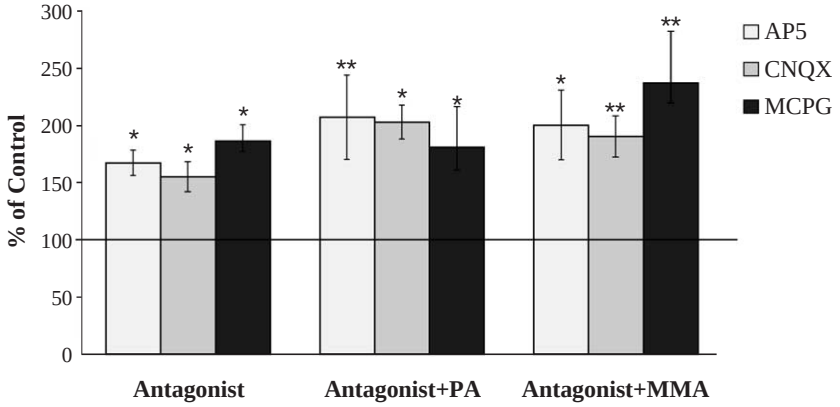

Fig. 2. Effect of glutamate antagonists on cAMP levels in slices from the cerebral cortex of 17-day-old rats. Slices of cerebral cortex from 17-day-old rats were first preincubated with 100 μM of the NMDA antagonist DL-AP5, 50 μM of the AMPA and kainate antagonist CNQX or 100 μM of the metabotropic antagonist MCPG. When indicated, 2.5 mM propionic (PA) or methylmalonic (MMA) acids were added and incubation proceeded for 30 min. Data are reported as means±S.E. of 8–10 animals in each group and expressed as percentage of controls. Statistically significant differences from controls, as determined by the one-way ANOVA followed by LSD test, are indicated: *P<0.05, **P<0.01.

mimicked by 1 mM glutamate. Moreover, when brain slices were incubated with 1 mM glutamate plus 2.5 mM PA or 1 mM glutamate plus 2.5 mM MMA, the effect observed was not additive (Fig. 1), suggesting that PA, MMA and glutamate induced an increase of cAMP concentrations by a similar mechanism. On the other hand, 10 μM epinephrine, a classical adrenergic agonist, was also able to increase cAMP levels after 5 min incubation but failed to induce any effect after 15 min incubation (Fig. 1). We also verified that the induction of high cAMP levels was totally dependent on extracellular Ca^{2+}, since when tissue slices were incubated with PA, MMA or glutamate in a Ca^{2+}-free buffer at 30 min of incubation, these metabolites failed to increase cAMP levels (Table 1).

Next, we tested the influence of glutamate antagonists on the stimulatory effects of PA and MMA on cAMP levels. To our surprise, when tissue slices were incubated for 30 min with the glutamate antagonists, we observed that the iGluR antagonists AP5 and CNQX, as well as the mGluR antagonist MCPG by themselves, significantly increased brain cAMP levels (Fig. 2). In addition, the preincubation of cortical slices with the glutamate antagonists followed by the addition of PA or MMA did not change this effect, suggesting that PA, MMA and the glutamate antagonists increased cAMP levels through other mechanisms than glutamate receptor stimulation (Fig. 2). In addition, when tissue slices were incubated for 30 min in a Ca^{2+}-free buffer with all the glutamate antagonists tested, these metabolites failed to stimulate cAMP levels (Table 1).

Taking into consideration our previous findings that epinephrine also caused an increase in cAMP levels (Fig 1) and considering that cAMP levels are closely related to β-adrenergic stimulation, we also tested the effect of the β-adrenergic antagonist propranolol on the induced increase of cAMP levels by PA, MMA and glutamate. Results showed that propranolol by itself had no effect on cAMP levels at different incubation times and, besides, it prevented the stimulatory effects of PA, MMA and glutamate at 30 min incubation, strongly suggesting that β-adrenergic receptors are involved in these effects (Table 2). Taking into account the previous data suggesting that the stimulatory effect of PA, MMA and glutamate on cAMP levels may have occurred via β-adrenergic mechanisms, we tested whether propranolol could also prevent the effect of the glutamate antagonists on cAMP levels. Initially, we tested the separated effect of each glutamate antagonist on cAMP levels at the three incubation periods studied. Results showed that AP5 was not able to stimulate cAMP levels at short incubation times (5 and 15 min) (Fig. 3), but elicited a significant increase of cAMP concentrations at 30 min incubation, as shown in Fig. 2. In contrast, CNQX and MCPG increased cAMP levels at all incubation times studied (Fig. 3). It can be also seen in Fig. 3 that propranolol

Table 2
Effect of propranolol on the increase of cAMP levels provoked by propionic acid, methylmalonic acid and glutamate in the slices of cerebral cortex from 17-day-old rats

	5 min (%)	15 min (%)	30 min (%)
PA	120±6.67	130±6.55	197**±25.67
MMA	110±7.90	98±10.50	193**±22.28
GLU	108±5.39	86±6.15	209**±26.39
PRO	89±5.80	92±13.30	116±14.30
PRO+PA	84±9.30	112±15.30	119*±18.60
PRO+MMA	74±21.10	75±15.40	105[a]±21.70
PRO+GLU	–	–	116[b]±19.20

Results are reported as mean±S.E. of eight animals in each group and expressed as percentage of controls (100%). Statistically significant differences from controls, as determined by one-way ANOVA followed by LSD test, are indicated.
Propionic acid=PA; methylmalonic acid=MMA; propranolol=PRO; glutamate=GLU.
[a] P<0.001 (MMA-treated slices as control).
[b] P<0.001 (GLU-treated slices as control).
* P<0.001 (PA-treated slices as control).
** P<0.001 (untreated slices as controls).

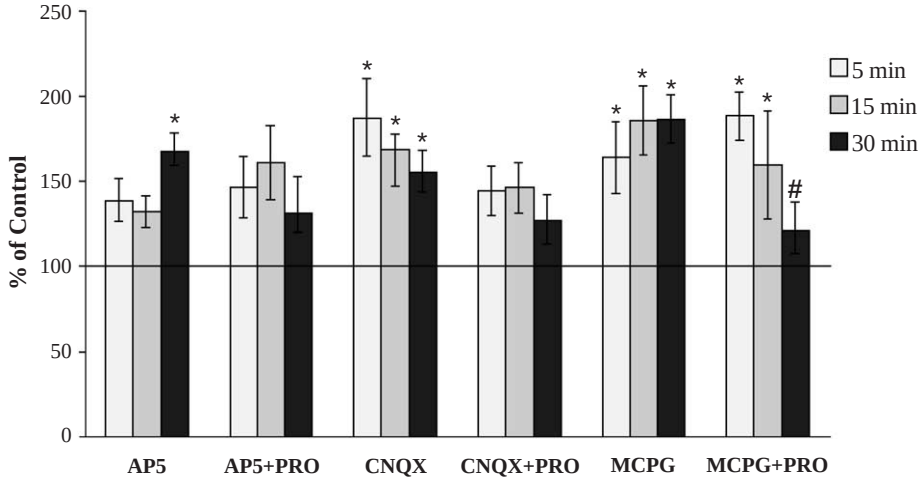

Fig. 3. Effect of glutamatergic and adrenergic antagonists on cAMP levels in the slices from the cerebral cortex of 17-day-old rats at different incubation times. Tissue slices were preincubated and incubated with 100 μM of the NMDA antagonist DL-AP5, 50 μM of the AMPA and kainate antagonist CNQX, 100 μM of the metabotropic antagonist MCPG in the presence or absence of 10 μM of the adrenergic antagonist propranolol (PRO) during 5, 15 or 30 min. Results from 8 to 10 different animals were obtained as described in Materials and methods and for each animal the control samples were referred to as 100%. Data are expressed as mean±S.E. Statistically significant differences, as determined by one-way ANOVA, followed by LSD test, are indicated. Different from control: *$P<0.05$. Different from MCPG (30 min of incubation): $^{\#}P<0.01$.

prevented the effect induced by the mGluR antagonist MCPG after 30 min of incubation, suggesting that the mechanisms by which MCPG elicit cAMP synthesis is somehow dependent on the β-adrenergic receptor, whereas those induced by AP5 or CNQX seem to be independent of adrenergic stimulation.

4. Discussion

We have previously demonstrated that PA and MMA increased the in vitro phosphorylation of cytoskeletal proteins in rat cerebral cortex through cyclic AMP-dependent protein kinase and that this effect was dependent on glutamate receptors [1,2]. In the present study we demonstrated that PA, MMA and glutamate elicit a significant increase of cAMP levels in slices of rat cerebral cortex in the presence, but not in the absence of calcium. These findings may reflect the expression of type I adenylyl cyclase in the cerebral cortex, which is activated by Ca^{2+} and calmodulin [3].

We also verified that the effect of PA and MMA on cAMP was mediated by β-adrenergic mechanisms. This is supported by the observation that the β-adrenergic non-selective antagonist propranolol prevented the stimulatory effects of these metabolites, and of glutamate on cAMP levels. Accordingly, the presence of a presynaptic β-adrenergic receptor linked to the cAMP pathway in the cerebral cortex has been described [13]. In addition, it is well known that the β1- and β2-adrenergic receptors are Gs-coupled proteins and that their stimulation causes an elevation of the second messenger cyclic AMP [31]. It should be however stressed that propranolol has also a partial antagonist effect on 5-HT1,2 serotonin receptors, so a serotoninergic modulation involving Gi/AC/cAMP- and Gq/PLC/IP3/Ca^{2+}-dependent pathways may also be implicated in the phenomenon observed [32,33].

We also observed that epinephrine, a β-adrenergic agonist, stimulated cAMP production at 5 min exposure, but not afterwards, while PA, MMA and glutamate required 30 min to induce a similar response. The probable loss of sensitivity of the adrenoceptor to epinephrine after 5 min incubation could be possibly due to desensitization, which frequently involves receptor phosphorylation/dephosphorylation cycles by G protein-coupled receptor kinases (GRKs) [34]. In this context, it has been described that protein kinase A (PKA) phosphorylates β_2-adrenergic receptors inducing the uncoupling and desensitization of this receptor [31]. Otherwise, the sensitivity of the β-adrenergic receptor to glutamate, PA and MMA could be related to the recently described molecules that interact with various GPCRs, mediating distinct signaling mechanisms [35].Thus, Shih and colleagues [36] showed that gravin, a prominent member of AKAP family of anchor/scaffold proteins, interacts with the β_2 adrenergic receptor in human epidermoid carcinoma cells, regulating the recycling of this receptor after agonist-induced desensitization. Since the effect of glutamate, PA and MMA involved β-adrenoceptor resensitization, it is tempting to speculate that gravin, in association with protein kinases and phosphatases, could modulate the recycling of β-adrenoceptor. Additionally, increasing evidence points to GPCR interactions between neurons and glial cells, via adrenoceptors, adenosine, dopamine and muscarinic receptors [37].

It is important to emphasize that our experimental model using cortical slice preparations for measuring cAMP levels does not allow to evaluate the evoked release of different neurotransmitters from neuronal and glial cells.

It is known that the mechanisms involved in neuronal and glial regulation often show counterbalancing changes in cAMP formation. It has been recently shown in astrocytes that the elevation of stimulated cAMP production can be induced by glutamate agonists acting at group II mGluRs and this potentiation is also altered by group I mGluR stimulation and extracellular Ca^{2+} levels via diacylglicerol–inositol 1,4,5-triphosphate/phospholipase C (DAG-IP3/PLC)- and Ca^{2+}/calmodulin kinase II (CaMKII)-dependent pathways [25].

It should be also emphasized that the increased cAMP levels may result in the production and release of endogenous adenosine (AD), which in turn acts at Gs-protein-coupled A_{2A} adenosine receptors, stimulating the release of excitatory neurotransmitters. The interaction of AD with glutamate receptors is also evidenced by directly inducing the release of AD when glutamate receptors are stimulated with glutamate, NMDA, kainate or quisqualate [38]. These elevated intracellular cAMP levels result in the release of endogenous AD, which in turn acts at Gs-protein-coupled A_{2A} AD receptors. In addition, extracellular AD raised by intra- or extracellular cAMP degradation can also act on A_1 AD receptors, which modulate voltage-dependent Ca^{2+}-channels (VDCCs) [39].

We have previously described that PA and MMA increased PKA-dependent phosphorylation of cytoskeletal proteins [2]. Taking into account that in the present report we observed that the effects of PA, MMA and glutamate on cAMP levels are Ca^{2+}-dependent, we could speculate that the activation of calcium-dependent adenylyl cyclases participates in this effect. This possibility is corroborated by the observations that the effects elicited by PA, MMA and glutamate on cAMP were not additive, suggesting a similar mechanism. On the other hand, altered intracellular cAMP levels are able to interfere with Ca^{2+} entry through voltage-dependent and/or receptor-modulated ion-channels, modulating further cAMP accumulation and regulating the consequent glutamate release from cerebrocortical [40] and corticostriatal [41] glutamatergic nerve endings via excitatory GsPCR/AC/cAMP/PKA-linked and/or inhibitory Gi/VDCC/Ca^{2+}-linked signaling cascades [37]. This possible cross-talk of different signal transduction pathways could contribute to the increased cAMP levels in response to treatment with PA and MMA.

Surprisingly, we also observed that, besides not preventing the increase of cAMP induced by MMA and PA, the glutamate antagonists CNQX and MCPG by themselves induced increased cAMP concentrations at all incubation times studied (5, 15 and 30 min), while the NMDA antagonist AP5 increased cAMP only after 30 min incubation. Furthermore, when tissue slices were incubated with each of the glutamate antagonists studied in a Ca^{2+}-free buffer no effect on cAMP levels was observed, again suggesting a mechanism dependent on extracellular calcium. On the other hand, considering that propranolol was effective in preventing only the effect induced by the mGluR antagonist MCPG after 30 min incubation, it is conceivable that the mechanisms by which these antagonists interfere with cAMP synthesis are not similar. However, to date, we do not know how glutamate antagonists increase cAMP levels, although these and further experiments are necessary to clarify this matter.

The concentrations of MMA and PA inducing in vitro alterations of cAMP levels in the present study are similar to those found in the cerebrospinal fluid of patients with MMAcidemias, which usually reach 3 mM or higher concentrations during metabolic decompensation [42]. In conclusion, the present study demonstrates that MMA and PA increased the production of the important intracellular second messenger cAMP probably via a β-adrenergic/G-protein pathway in brain cortical slices from developing rats. Even though the exact mechanisms underlying such effects are not completely known, we may propose that an increase of cerebral concentrations of cAMP may activate other signaling pathways that are involved in the neurotoxicity of PA and MMAcidemias.

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